The Effect of Oxygen and Carbon dioxide On the Development of Certain Cold Blooded Vertebrates

By

Ada Roberta Hall

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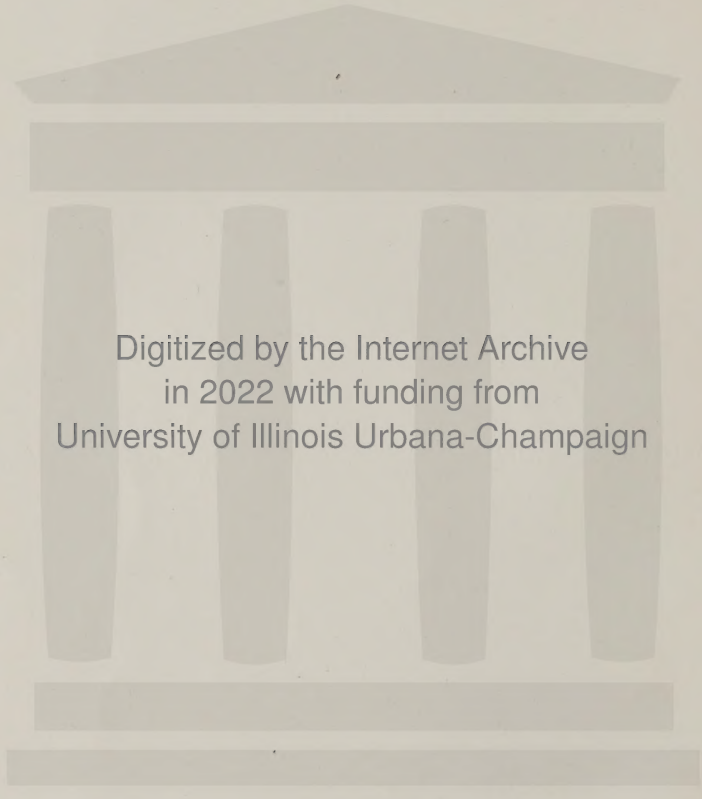
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THE EFFECT OF OXYGEN AND CARBON DIOXIDE ON THE DEVELOPMENT OF EGGS OF THE TOAD, *BUFO AMERICANUS*[1]

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INTRODUCTION

The success of a species in a given habitat depends largely upon the part which environment plays in the development of the individual, and the location of the most sensitive periods in the life history. The toad, *Bufo americanus* Le Conte, lives and breeds in warm, temporary, stagnant pools, thick with suspended mud and decaying matter, subject to many fluctuations in temperature and all other environmental factors, while the whitefish,[2] must have clean cold water comparatively free from decaying materials, presenting the minimum of daily fluctuations. This paper is an attempt to determine the effects of variations in dissolved gases, of a magnitude common in the toad habitat, upon development, and to locate the sensitive stages in the early life history. An index of sensitivity was also sought in the amount of oxygen released from hydrogen peroxide.

MATERIALS AND METHODS

It was found that the toads at Urbana, Illinois, breed in a variety of places from barnyard pools thick in manure and mud, to temporary pasture puddles. A plentiful supply also breed in a small artificial lily pond on the campus of the University of Illinois where the adults may be easily captured. They begin laying early in the morning and will continue until noon or after when brought into the laboratory. This affords a continuous supply of freshly fertilized eggs throughout the morning, and is more satisfactory than artificial insemination. A further advantage that these eggs possess for such a study as this is the fact that the time from fertilization to the functioning of the internal gills at 16° C. is very short, about twenty days. This makes it possible to watch their growth from hour to hour and to see the effect of changing the conditions. The eggs are large enough to see the stages well under a hand lens.

The pH was determined by the use of Hynson, Westcott and Dunning indicator sets with additional tubes for high and low values. The oxygen determinations were made by the Winkler method. As the amounts of

[1] Contribution from the Zoological Laboratories of the University of Illinois. 244

[2] Studied for comparison, results to be published later in ECOLOGY.

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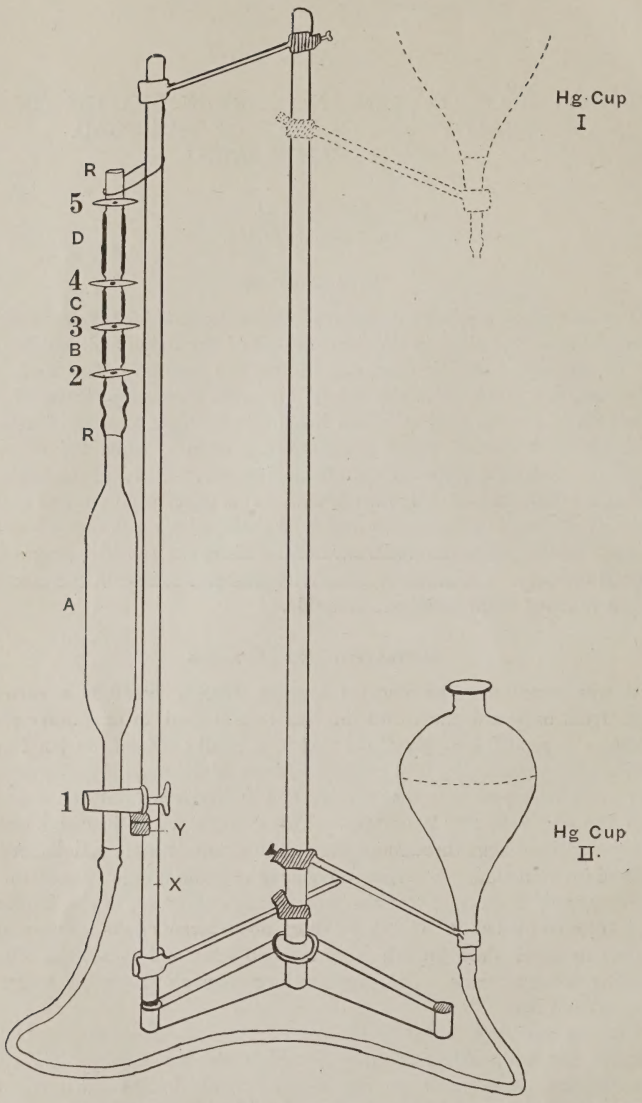

FIG. 1. Apparatus for measuring the oxygen content of small amounts of water by the Winkler method. **A**, large tube 2.2 cm. diameter by 6.5 cm. length, capacity 26 cc., total length from 1 to 2 is 17 cm. **R** to **R** heavy black rubber tubing enclosing glass rings **B C** and **D**, diameter 1 cm. outside, .6 cm. inside.

water in the experimental dishes were small, 200 to 400 cc., the use of 250 cc. bottles was impracticable. A piece of apparatus was therefore devised that would handle small amounts of water (Fig. 1). By raising and lowering the mercury cup, water was siphoned into the apparatus from the experimental dish, and the cocks 1 and 2 closed. **B** was filled with manganous chloride (sol. I, Winkler method), **C** with potassium hydroxide-iodide (sol. II), and **D** with concentrated hydrochloric acid, and clamps 3, 4, and 5 closed after each addition. The clamps were then removed one at a time in the same order and the liquids mixed by means of gravity, and a glass bead inserted with solution I. The 26 cc. of water which the apparatus holds was then titrated against N/40 sodium thiosulphate and the oxygen in cc. per liter calculated.

Experimental Data

A. Physiological Life History from Fertilization to Internal Gill Stage

In this work on physiological life history several types of experiments were performed: (1) Keeping the pH constant (8.0) the oxygen content was varied, four concentrations, .4 cc., .9 cc., 1.41 cc. and 4.64 cc. per liter being used. (2) Keeping the oxygen about the same in all dishes, .4 cc. to .8 cc. per liter, the pH was varied from 6.1 to 8.0. (3) Eggs were sealed into

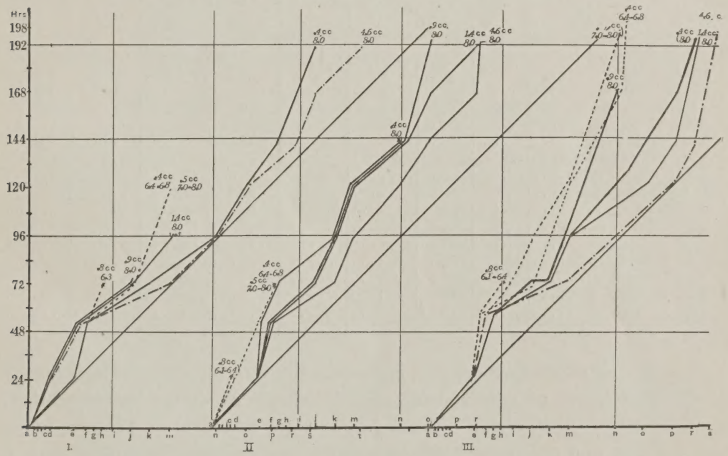

Fig. 2. Toad eggs put into experimental conditions: I at 1 cell stage, II 2 cell stage, and III 4 cell stage.

a fertilization, **b** 2 cell, **c** 12–16 cell, **d** early blastula, **e** late blastula, **f** blastopore lip, **g** yolk plug large, **h** yolk plug medium, **i** yolk plug small, **j** early neural folds, **k** late neural folds, **m** elongation, **n** tail differentiating, **o** tail 1/4 body, **p** tail 1/3 body, **r** tail 1/2 body, **s** tail 2/3 body, **t** tail equal to the body (7 days), **u** 8 days, **v** 9 days, **x** point of death.

known quantities of water with differing oxygen content and the time and extent of development noted.

Time for normal development of toad eggs at 16° C., plotted as a straight line in graphs of figs. 2 and 3, is as follows: Fertilization 0 hrs.; first cleavage (2 cell), 3 hrs. and 20 min.; 4 cell, 4 hrs. and 30 min.; 8 cell, 6 hrs.; 12 to 16 cell, 8 hrs.; early blastula, 10 hrs. and 30 min.; late blastula, 23 hrs. and 30 min.; blastopore lip, 28 hrs.; yolk plug-large, 31 hrs. and 30 min.; yolk plug medium, 41 hrs.; yolk plug small, 48 hrs.; early neural folds, 51 hrs.; late neural folds, 62 hrs.; elongation, 72 hrs.; out of jelly—tail differentiating, 95 hrs.; tail 1/4 length of body, 114 hrs.; tail 1/3 length of body, 123 hrs.; tail 2/3 length of body, 143 hrs.; tail equal to body, 168 hrs.; gills three, 192 hrs.

TABLE I. *Time in hours for reaching various stages of development in the toad experiments*

Read down the columns; embryos died where the number is underscored.

	Series I. Put in 25 Min. before 1st Cleavage				Series II. Put in at 2 Cell Stage			
	A–1	*A*–4	*C*–1	*C*–2	*A*–1	*A*–4	*C*–1	*C*–2
pH	8.0	8.0	5.8–6.6	6.0–6.4	8.0	8.0	5.8–6.6	6.0–6.4
Oxygen Content (c.c.)	.4	4.64	.8	.4	.4	4.64	.8	.4
Time from fert. to:								
1st cleavage	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Early blastula	.....	24	.....	.....	.....	.....	24	24
Late blastula	24	56	24	24	24	24	.....	56
Blastopore lip	56	.....	56	56	60	56	.....	.....
Yolk plug large	.....	.....	.....	.....	73	.....	.....	73
Yolk plug medium	.....	.....	73	.....	.....	.....	.....	.....
Early neural folds	.....	.....	.....	73	.....	.....	.....	.....
Late neural folds	73	.....	.....	95	95	73	.....	.....
Elongation	.....	73	.....	123	123	95	.....	.....
Tail differ	95	95	.....	.....	143	125	.....	.....
Tail 1/4 body	123	123	.....	.....	.....	143	.....	.....
Tail 1/3 body	143	.....	.....	.....	.....	.....	.....	.....
Tail 1/2 body	168	143	.....	.....	.....	168	.....	.....
Tail 2/3 body	192	168	.....	.....	.....	.....	.....	.....
Tail = body	.....	192	.....	.....	.....	.....	.....	.....

In the first type of experiment eggs were put into the experimental dishes at different stages of development, and the effects as shown by retardation, acceleration or death noted. These results were tabulated in Table I, while the *A* series and the same results are shown graphically in figures 2 and 3. In making the graphs, normal development was taken arbitrarily as a straight line from fertilization at 0 hours to the internal gill stage at 196 hours. Time in hours was plotted as ordinates, and the stages of development, or abscissae, were obtained by dropping perpendiculars from the normal development line to the x axis according to the number of hours required to

reach that development. All experimental curves were then drawn with both time and period of development fixed by the normal curve. When the experimental curve rises above the normal line retardation is indicated; when it drops toward the normal or below it, acceleration is indicated. Curves parallel to the normal denote normal rate of growth.

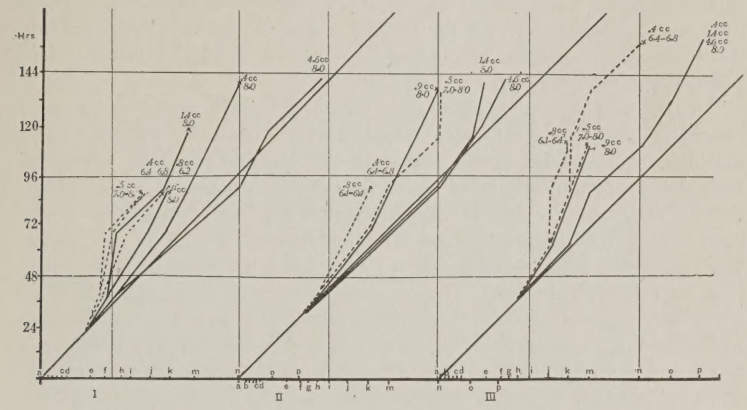

FIG. 3. Toad eggs put into experimental conditions: I at late blastula, II at yolk plug, and III at blastopore lip stages. Notation same as Fig. 2.

Using a pH of 8.0 and four concentrations of oxygen, .4 cc., .9 cc., 1.41 cc., and 4.64 cc. per liter, we find that a certain degree of development was attained regardless of the concentration, death not occurring in any of the experiments short of the yolk plug stage even when the oxygen was as low as .4 cc. per liter. Eggs put in before the first cleavage (Fig. 2–I) show marked retardation up to the late blastula stage then some acceleration so that further development approaches normal. Eggs exposed to the experimental conditions between the first cleavage and the late blastula stage (Figs. 2 and 3) show a period of eighteen or twenty hours with little or no retardation and then a period of quite marked retardation occurring in the blastopore or yolk plug stages. Later development for the higher concentrations of oxygen again approaches normal. Eggs put in after the late blastula stage (Fig. 3) show general retardation for the lower oxygen concentrations but fairly normal development for the higher concentrations.

The results of this type of experiments show that where the oxygen was as low as .4 cc. to .9 cc. per liter death occurred in part of the experiments, but at higher concentrations only retardation showed the effects of exposure. At the late blastula and blastopore lip stages the higher concentrations even caused acceleration of growth. The most sensitive stages appeared to be from fertilization to the first cleavage, and the gastrulation stages.

The second type of experiment was where the oxygen was low (.4 to .8 cc. per liter) and the pH varied, 6.1, 6.4, and 7.0 to 8.0. The stages reached in development are shown in the graphs of Figs. 2 and 3. Exposures to these conditions beginning before the four cell stage gave death with retardation in 24 to 120 hours, *c* to *k*. The same effect is seen in eggs put in after the eight cell stage. Those exposed between the four and eight cell stages show the greatest development (198 hrs.) which is past the sensitive stages. With small amounts of oxygen the effect was much more rapid and deadly with a pH of 6.1 to 6.4 than with a pH of 6.4 to 6.8.

In the third type of experiment a dozen eggs were placed in an eight dram vial (about 26 cc. of water) supplied with a two-holed stopper having the inlet tube extended to the bottom of the vial and the oulet tube even with the stopper. Both tubes ended outside the stopper in short rubber tubes. Water of the desired oxygen content was run through the bottle until all oxygen due to air was flushed out and then a clamp on the two rubber tubes sealed the bottle. Development was watched by means of a dissecting lens until growth stopped. The oxygen content was then determined by adding the chemicals through the inlet tube, being careful to avoid air bubbles when inserting the pipettes in the rubber tubes. The cleavage of a dozen eggs proceeded to the late blastula stage regardless of the period of development when put in or the amount of oxygen present (.1–5.7 cc. per liter). Further development was roughly proportional to the amount of oxygen present, as was also the time to death.

B. Reaction of Eggs to Hydrogen Peroxide as Development Progresses

A universal property of protoplasm (both plants and animals) is the abitilty to liberate oxygen from hydrogen peroxide. What this property is due to is a debated question at present, but in general it is ascribed to an enzyme called catalase. Several theories have been advanced as to its significance in animal life. References are given to these in the literature cited (Becht '19, Burge and Burge '21, Zieger '15). As a measure of the sensitivity of the developing egg at different stages in the early life cycle the reactions to oxygen and carbon dioxide (acidity) have been discussed. It was thought that a study of the amounts of oxygen released from hydrogen peroxide at various periods of development might be of significance. Accordingly a series of determinations was made with the toad egg using the method outlined by Burge ('16), Fig. 4**A**. A second series was made for the toad eggs the following spring using eight dozen eggs instead of one dozen and shaking the material by machine instead of by hand (Fig. 4**B**). A comparison of **A** and **B** shows a striking parallelism and the two curves seem to confirm each other.

From a study of these curves (Fig. 4) it may be seen that there was a decrease in the power of liberating oxygen from hydrogen peroxide from the

unfertilized egg to the fertilized, an increase from fertilization to the early blastula, a decrease from early blastula to elongation of the embryo, and from this point to twenty-one days a steady increase. The stages most sensitive to low oxygen and high acidity were (1) fertilization to first cleavage, and (2) the gastrulation stages. These were the low points on this curve. Is there a correlation between sensitivity and a lowered power of liberating oxygen from hydrogen peroxide?

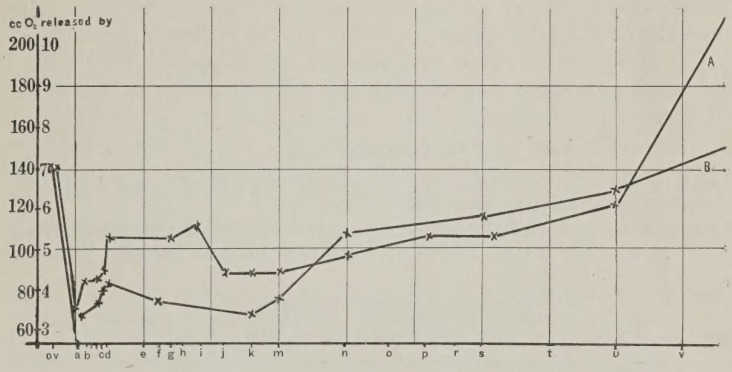

FIG. 4. Amounts of oxygen released from hydrogen peroxide at different stages of development of toad eggs. Series **A** determinations were made with one dozen eggs in 1920. Series **B** was made using eight dozen individuals in 1921.

Winternitz and Rogers ('10) have shown a definite increase in what they call catalase for the different stages of the hen's egg, as development proceeds. Burge and Burge ('21) have shown an increase in this power from egg to adult for the Colorado potato beetle. Zieger ('15) has found a definite rythm for catalase in the insect life history, reporting that the power is high where rapid growth or metamorphosis is going on (early larval stages, pupal stages) and low during resting stages. Child in his book "Senescence and Rejuvenescence" has shown that metabolism is high during the more sensitive stages. Burge believes that increase in catalase brings about an increase in metabolism. This curve for the toad egg seems to be a contradiction of one statement or the other since it shows the power of splitting oxygen from hydrogen peroxide to be low at the sensitive stages. More work is needed on complete life histories before definite conclusions can be drawn about this point.

I wish to thank Dr. V. E. Shelford, at whose suggestion this work was undertaken, for his helpful advice and kindly criticism during the course of the investigation. I am indebted to Dr. W. E. Burge and Dr. H. B. Lewis for suggestions in the enzyme work.

Summary and Conclusions

1. Conditions of high carbon dioxide or low oxygen content such as may occur in the normal environment and which are tolerated by the other periods of development, cause great retardation and even death at sensitive stages. The effects of low oxygen are much more detrimental in acid than in alkaline water.

2. There are two definitely sensitive stages in the early physiological life history of the toad (*a*) one cell stage; fertilization to first cleavage, (*b*) the gastrulation stages; blastopore lip formation to the small yolk plug stage.

3. There is a decrease in the amount of oxygen released from hydrogen peroxide at the sensitive periods which roughly corresponds in magnitude to the decreased resistance.

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EFFECTS OF OXYGEN AND CARBON DIOXIDE ON THE DEVELOPMENT OF THE WHITEFISH[1]

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Introduction

A subject of vital interest is the rhythm of events in the physiological life history of any species, and the manner in which this rhythm may be affected by environmental factors. Accordingly this work has been undertaken for the purpose of: (1) finding out the relative sensitivity of the stages in the early life history of the Whitefish; (2) testing the resistance and reactions of normally hatched individuals as compared with the reactions of those hatched under experimental conditions.

Materials and Methods

The material for this work was the lake whitefish, *Coregonus clupeiformis* Mitchill. Some work was done on fertilization and early cleavage stages at the U. S. Hatchery, Put-in-Bay, Ohio. The major part of the work, however, was done at the Vivarium, University of Illinois, on material shipped from the hatchery in ice. The temperature of the lake water was about 8° C. at the beginning of the season. It had a pH of 7.0 and an oxygen content of 4.08 cc. per liter. At the Vivarium the stock was kept in water from the University wells aerated to about 2.6–3.3 cc. per liter, and with a pH of 7.8. It was cooled to 10° C. by means of brine coils. The water (pH 8.0–9.0; carbonates 232 ± parts per million) for all the experiments was boiled free of all dissolved gases, and part of the salts precipitated (Shelford '18).

The apparatus used for varying the pH and oxygen content is shown in figure 1. Bottle 1 contained approximately N/4 sulphuric acid which siphoned over into the mixing bottle 4. The stopcock 2 controlled the flow which could be measured by counting the drops through the glass bulb 3. Thus a known amount of the acid was added to a known flow of the boiled water which entered at *W*. This water was also 10° C. The water leaving 4 had a known pH, and was oxygen free. Since it contained carbonates, acids set free carbon dioxide which changed the pH. The oxygen was controlled by adding compressed air (see fig. 1 and explanation). Three such sets of apparatus were used giving three hydrogen ion concentrations, each with three oxygen concentrations.

[1] Contributions from the Zoological Laboratory of the University of Illinois, No. 256.

The pH was determined by the use of Hynson, Westcott, and Dunning indicator sets with additional tubes for high and low values (Clark '20). Brom cresol purple (5.8–6.6), brom thymol blue (6.6–7.6) phenol red (6.6–8.0), and thymol blue, alkaline range (8.0–9.2) were used. The colorimetric

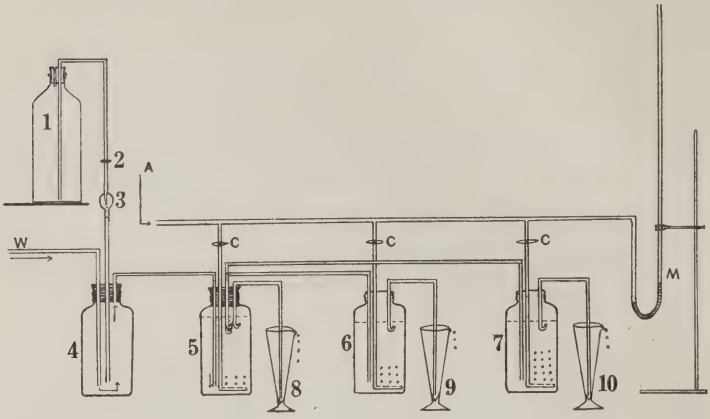

Fig. 1. Apparatus for the control of experimental conditions. Method of varying hydrogen-ion concentration and oxygen content. **1**, N/4 sulphuric acid; **2**, cock for control of acid by drops at **3**; **4**, mixing bottle for acid and boiled water from **W**; **5**, **6**, and **7**, jars, to which air from **A** is added in varying amounts; **C**, cocks for control of air flow; **8**, **9**, and **10**, half-pint sedimentation glasses for eggs; **M**, mercury manometer for keeping air flow constant.

standards were checked electrometrically by Dr. R. E. Greenfield. Oxygen determinations were made by the Winkler method with an apparatus which used only 26 cc. of water for a determination (Hall '23).

Experimental Data

Experiments at the Hatchery

All the experimental work was done at 10° to 11° C. at the U. S. Hatchery. The length of life of eggs and sperm was tested. Three sets were run (*a*) dry eggs and sperm, (*b*) dry eggs and sperm mixed thoroughly with water to uniform milky fluid, and (*c*) dry sperm and eggs standing in lake water. With wet eggs and dry sperm eight minutes was the latest time at which fertilization was possible. With wet sperm and dry eggs nine minutes showed a small number of fertile eggs. But with both eggs and sperm dry fertilization took place at seven and a half hours. It may have been possible after longer time as such an experiment was destroyed and could not be verified.

In early development the eggs were exposed to constant oxygen (4.08 cc. per liter) but with varying pH. Normal lake water had a pH of 7.0. Acidity (pH 6.2–6.6) was produced by adding sulphuric acid, and alkalinity (pH 8.4–8.6) by adding sodium hydroxide. Dishes of standing water were used and changed frequently. Eggs fertilized directly in the solutions were normal at the end of twenty-four hours (32–64 cell stage). This experiment was then repeated using lake water boiled until the oxygen content was 2.9 cc. per liter. Fertilization and development occurred in all the dishes, but there was a marked difference between the acid and alkaline waters. With a pH of 6.2 and 6.6, respectively, 80 per cent and 25 per cent fertilized and developed; with a pH of 7.0 only 3 per cent, and at a pH of 8.4 but 1 per cent. A hydrogen ion concentration which is too great to favor later development appears best for fertilization (Cohn '18).

Experiments with Shipped Eggs

In the work on later stages with treated running water at the University of Illinois, special attention was paid: (1) to keeping conditions as near constant as possible from day to day; (2) to watching the stages of development reached in each concentration and comparing these with each other and the control stock; (3) to working out the death rate and the percentage hatching for each concentration; and (4) to testing the vitality and reactions of the larvae hatching from the different stocks.

In this work apparatus was set up in triplicate (fig. 1). Values of pH 6.4, 7.0, and 8.0–9.0 respectively were used, each pH with an oxygen content of 1 cc., 3 cc., and 4.5 cc. per liter; pH was taken in all the dishes each day, and oxygen was determined every two or three days.

The eggs were obtained from the hatchery in four lots. The first lot, spawned December 2, was in the thirty-two cell to early germinal cap stage when received December 3. The second lot, spawned December 5 and received December 24, had the tail just elongating and the fin buds starting. The eye vesicles had formed but no pigmentation had occurred. The third lot, spawned December 7 and received January 31, were only slightly farther along in development than lot two. They had just begun to show the pigment in the eyes. The fourth lot, received March 12, were fully developed and started to hatch immediately.

A comparison of the chemical analyses of Lake Erie water (the average of Huron at Port Huron and Erie at Buffalo, Clarke '20, p. 70) and the boiled University water is important in connection with the experimental results obtained with the whitefish, and is given in Table I.

The four stocks differed in: (*a*) the length of time in these two kinds of water, (*b*) temperature at hatchery and in experiments, and (*c*) the stage at which shipment was made.

TABLE I. *Comparison of Lake Erie and boiled University of Illinois water*

	Lake Erie Water	Boiled U. I. Well Water
pH (CO_2)	7.0	9.0
CO_3	54 ppm.	250 ppm.
SO_4	10 ppm.	trace
Cl	5 ppm.	1 ppm.
Ca	27 ppm.	33 ppm.
Mg	7 ppm.	27 ppm.
Na, K	5 ppm.	16 ppm.
Total solids	112 ppm.	248 ppm.

TABLE II. *Showing the development of whitefish eggs under different conditions*

Figures are days to stage indicated on left. Dates of spawning, receipt at Urbana, and beginning of experiment, respectively, follow each series number. Series II and III are stock 1; series VIII is stock 3. The entries in italics indicate that embryos died soon afterward. Oxygen all given in cc. per liter.

	A–1	B–1	C–1	A–2	B–2	C–2	A–3	B–3
Series II; 12/2; 12/3; 12/6.								
pH range	6.2–7.5	5.8–8.9	6.4–8.1	6.4–8.0	6.2–8.9	7.9–8.5	6.6–8.3	6.8–9.2
pH mean	6.2	6.2	6.4	7.0	7.2	7.2	7.8	8.0
Oxygen range	0–.9	0–1.04	0–2.08	0–.9	0–1.24	.01–3.7	0–.9	0–1.7
Oxygen average	.1	.2	.6	.1	.2	.7	.1	.2
Cap small cells	4	4	4	4	4	4	4	4
Embryo forming	*5*	5	5	*5*	5	5	*5*	5
Post ring large		*6*	6		6	6		
Post ring small	7	7	7	7	7	7	7	10
Eye vesicle								
Tail flat	*10*	10	9.5	*10*	10	9	*10*	10.8
Tail starting		12	11		11	10		11
Tail elongating		13	13		13	11		
Tail ¼ body						13		
Series III; 12/2; 12/3; 12/17.								
pH range	5.9–6.9	6.8–7.0	6.8–9.0	5.9–6.9	6.8–7.0	6.8–9.0	4.9–6.9	6.8–7.0
pH mean	6.4		8.5	6.4		8.5	6.4	
Oxygen range	.4–2.4	.4–2.2	.2–1.7	1.3–3.1	1.9–3.7	1.7–3.2	3.2–5.3	3.3–4.9
Oxygen average	1.64	.88	1.07	2.32	2.56	2.49	4.37	3.96
Pigmented	15	15	15	15	15	15	15	15
Fin rays short	19	19	17	*19*	*17*	17	19	17
Fin rays ½ fin			21				21	21
Tail near head	*26–27*	*26*	26			26		26
Tail to head			*27*			*27*	*27*	*27*
Tail to eye								
Hatching								
Series VIII; 12/7; 1/31; 2/21.								
pH range	6.1–7.2	6.2–9.0	9.0	6.1–7.2	6.2–9.0	9.0	6.1–7.2	6.2–9.0
pH mean	6.4	7.6	9.0	6.4	7.6	9.0	6.4	7.6
Oxygen range	.2–1.1	.1–.5	.8–2.4	2.0–3.2	.7–3.5	2.6–3.0	3.3–3.8	2.4–5.3
Oxygen average	.61	.4	1.6	2.6	2.12	3.0	3.5	3.8
Ready to hatch	76	76	76	76	76	76	76	76
Hatching—1st	78, 7%	78, 13%	78, 18%	78, 18%	78, 16%	78, 15%	78, 8%	78, 17%
Max. hatching	79–82	79–82	79	81–83	79–82	79–81	81–83	81–83

Effect of Conditions on the Different Stocks

Table II shows the number of days required to reach the different stages in the various concentrations. The numbers and letters used below will be found in this table.

Stock 1. In series II, conditions varied materially in all dishes. With a pH around 6.2–6.4 we find that a set of eggs put into the dishes *A, B* and *C*–1 on the seventh day varied in both amount of development and length of life roughly in proportion to the amount of oxygen supplied. This was .1 cc., .2 cc., .6 cc. per liter respectively. This amount was not enough to carry development beyond the elongation of the tail bud, and was lethal in every case. This same oxygen effect seemed to hold with higher pH, and was evidently below the threshold of whitefish development.

In series III, oxygen content was raised so that dishes No. 1 had about 1 cc. per liter, dishes No. 2 about 2.5 cc., and dishes No. 3 about 4 cc. per liter. With this increase in oxygen concentration the more alkaline water showed the farthest development. The eggs lived as long in the acid and neutral waters of high and low oxygen but development was retarded. It is significant that those eggs in neutral waters were more retarded than those in either the acid or alkaline waters. Where neutrality was maintained as in this set a detrimental effect is noticed in development equal to that of higher hydrogen ion concentrations. The results suggest that pH of 7.8 is optimum and 7.0 too high a concentration of hydrogen ions. This lot of eggs, put in at the early pigmentation stage of the eyes lived nearly to hatching.

Stock 2. The experiments of series IV and V were started three days apart and run simultaneously so that conditions were identical except for the time of entering the experimental waters. The average oxygen for the different dishes was slightly higher than in series III. The eggs of series IV were put in when the fin buds were just starting. A distinct oxygen differential was established: the low oxygen eggs developed to the point where the tail reached the mendian line of the head; the medium oxygen eggs to the point where the tail reached past the head and around to the eye; while the high oxygen eggs developed just to hatching in the acid and neutral waters. The alkaline water did not seem enough lower in oxygen to account for the retardation which occurred, and this retardation was probably due to the hydrogen ion concentration.

In series V more difference in development is apparent between the different dishes. The greatest amount of retardation occurred in the low oxygen jars and in the high oxygen of the neutral series (pH fluctuating). Two eggs hatched in the medium oxygen of the neutral series, but all others died just before hatching time. These were the first individuals to

hatch in the experimental dishes, though a few hatched in the stock (pH 7.8 and oxygen 3.3 cc. per liter).

Stock 3. Hatching occurred in the experiments run with both the third and fourth stocks. As these stocks were older, the time of exposure to the experimental waters was later, and a different reaction resulted. Series VI and VII were started two days apart but were run simultaneously. Hatching occurred in several of the dishes and extended over several days, the beginning and duration of the process being recorded in Table II. The per cent hatching is given as the second member opposite "Hatching-1st."

Series VIII was started just as the eggs of stock 3 were ready to hatch, to test the effect of different concentrations on this process and on the life of the larvae. Hatching occurred in all the dishes beginning on the same day. Maximum hatching was 2 days later in the acid "medium" oxygen and in the acid and neutral high oxygen, showing some retardation for these concentrations.

Stock 4. Series IX is the result of the exposure of eggs reared for the entire time of development at the hatchery in lake water. It shows the relative sensitivity of the hatching period and the newly emerged larvae. The first hatching occurred in the acid and neutral low oxygen an hour or two after putting the eggs in the water. A few also hatched in alkaline "low" and "medium" oxygen later the same day.

Differences in the Stocks at Hatching

The normal time for the development of these eggs at the hatcheries was four to five months. Due to higher temperature my first stock began hatching at thirty-two days. The newly hatched larvae from each of my four stocks were measured for body length, and for size and shape of yolk, and the time from hatching to death recorded.

Only a few individuals were measured in stocks 1 and 2. In stock 1 the fry were 8 to 11 mm. in length with round yolk sacs 2 mm. in diameter. These lived only two days. In stock 2 the fry were 11 mm. long, also with round yolk sacs. These lived sixteen days with no food. Stock 3 averaged 11.1 mm. with similar yolks and lived at least ten days. Stock 4, raised in lake water, gave fry of a distinctly different type. They were 13.4 mm. to 15 mm. long and were extremely active. The yolk sacs were oval but of the same volume as the earlier stocks. These lived for twenty-five days without food, and at the time of death measured 15 mm. This shows that eggs reared for a long period at low temperature attain a larger size and have more energy than they would have if forced at higher temperature (an argument against forcing conditions in hatchery work).

Mortality

Due to Fungus. One factor in mortality was fungus growth. This was in direct proportion to the amount of oxygen present, therefore was most detrimental in the high oxygen concentrations. The pH seemed to have no differential effect. The fungus grew very rapdily on dead eggs but also entangled live ones and killed them. Infected eggs left in the dishes would mat dozens of eggs together and kill them in a day or two. Since higher temperatures were more favorable to the fungus it grew more abundantly in my dishes than at the hatchery.

Due to Experimental Conditions. The eggs of the different series (II to IX) were counted and the dead recorded from day to day. From this data the per cent of deaths was calculated for each dish of the series. The stages from early formation of the embryo and ring seemed very sensitive, as in all concentrations the deaths quickly amounted to 100 per cent. From the time of closure of the posterior ring and beginning of elongation of the tail and fin buds sensitivity decreased, as shown by the following figures:

Series II: Early gastrulation. At the end of three days the per cent which had died was as follows: A-1, 100 per cent; B-1 100 per cent; C-1, 66 per cent; A-2, 100 per cent; B-2, 75 per cent; C-2, 50 per cent; A-3, 100 per cent; B-3, 95 per cent; C-3, 50 per cent.

Series III: Eye pigmented, lens showing. At the end of nine days the per cent which had died was as follows: A-1, 20 per cent; B-1, 20 per cent; C-1, 10 per cent; A-2, 50 per cent; B-2, 100 per cent; C-2, 5 per cent; A-3, 10 per cent; B-3, 50 per cent; C-3, 5 per cent.

Series IV: Tail 1/4 body length. At end of sixteen days the per cent which had died was as follows: A-1, 91 per cent; B-1, 26 per cent; C-1, 49 per cent; A-2, 64 per cent; B-2, 96 per cent; C-2, 98 per cent; A-3, 70 per cent; B-3, 66 per cent; C-3, 98 per cent.

The deaths due to fungus were in most cases small compared to total deaths. Total mortality is the index to the effect of varying conditions of oxygen and carbon dioxide, and shows the relative sensitivity of the different ages of eggs.

Effect of Other Acids

As sulphuric acid liberated the carbon dioxide in the above experiments, it was thought desirable to run a check with other acids. A series of finger bowls was prepared such that a pH of 6.3, 7.0, and 9.0 was obtained for each, sulphuric, hydrochloric, and acetic. Salts such as sodium chloride antagonize the acid which may be present in water (Loeb, '12, Osterhout, '14), and therefore a duplicate series was made up in which one third of the volume of water was substituted by M/6 sodium chloride. The same amount of

mixing was given each solution as it was made up to insure a uniform oxygen content of 1.5 cc. per liter. The finger bowls were filled full and covered. Readings were taken without uncovering. Table III shows the time to death in the different concentrations. In the acetic and sulphuric acid (no salt) death occurred in pH 7.0 first, 9.0 second and 6.3 last, but for hydrochloric, death in 6.3 preceded 7.0. Neutrality was most quickly fatal, and alkalinity more toxic than acidity. The sulphuric plus salt was least toxic of all with a pH of 6.3, as these larvae were still alive at the end of 23 days. The differ-

TABLE III. *Resistance of whitefish larvae to acidity, neutrality, and alkalinity in 500 cc. fresh boiled water and M/6 NaCl water; acid, acetic 0.529N sulphuric 0.485N, hydrochloric 0.442N, CO_2 gas added directly*

No. Fish	Chemical Used	O_2 in cc. per L.	pH	Av. Time to Death	Remarks
4	.8 cc. acetic + boiled H_2O	1.5	6.3	47 hrs.	
4	.8 cc. " + M/6 NaCl	"	"	84 "	
4	.18 cc. " + boiled H_2O	"	7.0	22 "	
4	.18 cc. " + M/6 NaCl	"	"	84 "	
4	.6 cc. HCl + boiled H_2O	"	6.3	20 "	
4	.6 cc. " + M/6 NaCl	"	"	180 "	
4	.26 cc. " + boiled H_2O	"	7.0	51 "	
4	.26 cc. " + M/6 NaCl	"	"	61 "	
4	.6 cc. H_2SO_4 + boiled H_2O	"	6.3	52 "	
4	.6 cc. " + M/6 NaCl	"	"	216 "	1 alive 24 days
4	.16 cc. " + boiled H_2O	"	7.0	27 "	
4	.16 cc. " + M/6 NaCl	"	"	500 "	1 alive 24 days
4	control boiled water	"	9.0	47 "	
4	" M/6 NaCl.....	"	"	87 "	
24	CO_2 + boiled water	.1	6.3	2 hrs. 25 min.	Stock 3
45	" + " "	.1	6.3	4 "	" 4
15	boiled water	.1	9.0	3 " 45 "	" 3
9	" "	.1	9.0	5 "	
15	CO_2 + boiled water	2.8	6.4	5 " (not dead)	" 3
18	" + " "	2.8	6.4	12 " " "	" 3

ence in the reactions to the three acids was small. The variation in the hydrogen ions was probably influencing growth rather than the anions, sulphate, acetate, and chloride. This is in keeping with the work of Loeb ('04, '12) on the chemicals most influencing growth. He has studied the effect of salt solutions on growth and regeneration and finds that the cations or substituted hydrogen ions are most influential in changing growth, calcium, sodium, and potassium being necessary at some time during the life cycle for normal individuals to develop.

The effect of direct addition of carbon dioxide to water of high and low oxygen was studied in relation to length of life in whitefish larvae. In low oxygen the larvae died more rapidly in acid water (6.3) than in alkaline

(9.0). With high oxygen (3 cc. per liter) a pH of 6.3 did not kill, though the exposure was for the greater part of two days. This is in accordance with Well's statement that large amounts of oxygen antagonize the detrimental effects of high concentrations of carbon dioxide. Statements of the effect of the different combinations of oxygen and carbon dioxide in this paper seem contradictory. The experiments were all started with different stocks so the number of items that can be compared is unavoidably small. However when the time to "fin ray short" (series III, table II) is plotted on cross section paper with oxygen and pH scales, and the points showing equal time connected in a manner suggested by Huntington's ('19) plots of human death rate in relation to temperature and humidity, the results appear orderly (Fig. 2). The time for "tail flat" in series II shows a similar

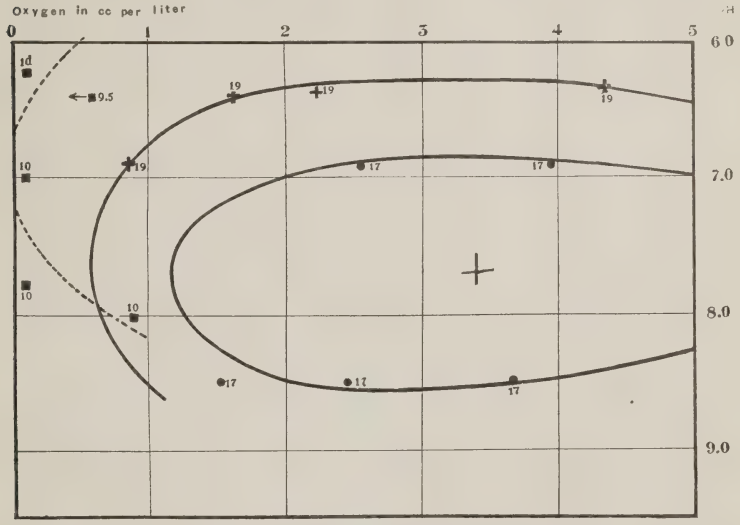

Fig. 2. Showing equal time lines on an oxygen-hydrogen-ion chart. The broken line passes approximately through combinations of oxygen and hydrogen-ion concentration in which the embryos developed a flat tail in ten days, beginning four days after spawning. The solid lines pass through combinations of oxygen and hydrogen-ion concentration in which the embryos developed short fin rays in 17 and 19 days respectively. The general trend of these lines suggests that development may be expected to be most rapid at about 3.4 c.c. of oxygen and pH 7.6 to 7.7, where the large cross is placed. This cross is in the center of the ellipse. The center of the ellipse suggested by the flat tail curve would fall at a higher H-ion concentration.

relation. Observations were not made often enough to bring out the necessary details in any but series III, as noted above; accordingly this interpretation can be suggested only as a basis for further investigation.

Under experimental conditions eggs develop into normal embryos with a pH of 7.0 and a much lower oxygen content than in the hatchery. Much greater variations were produced in per cent hatching and in length of time for development by varying the pH toward the acid or alkaline end of the scale than by changing the oxygen. This is in accord with the idea of Powers ('20). He concluded from his reconnaissance of the pH of Puget Sound waters under varying conditions of tides, weather, *etc.*, that pH has more to do with compatibility of habitat than oxygen.

Effect of Conditions on Reactions of Fry from Different Stocks

As each of the stocks hatched, gradient experiments were run with the larvae to see if the environment during the early embryonic life had any effect on the pH range which they would choose. A gradient tank suited to the size of the larvae was used. This tank was 20.5 cm. by 2 cm. by 1.5 cm. with screens 1.5 cm. from each end, and outlets at both sides of the middle. Water was introduced drop by drop behind the screens at the ends, and flowed out at the center. The end thirds were thus very nearly the same as the water introduced there, while the center third was a mixture of the two.

Stock 1 and 2 were reared from the early germinal cap stage and the elongation of the tail, respectively, in the vivarium waters. Both of these stocks then lived through the *period* of *heart* and *blood formation* in the experimental waters. The results of the gradient experiments are suggestive. No larvae hatched from the experimental dishes of the first stock, but those hatching in the control (pH 7.8) stayed within the range of 7.6 to 8.2 when given a choice of 6.6 to 9.0. Fig. 3 *A* shows the reactions of larvae of stock 2 to control water (7.8). When given a gradient of 6.4 to 8.2 a very definite choice of end is seen. Larvae reared in water of moderate hydroxyl ion concentration (pH 9.0, Fig. 2 *B*) choose the alkaline end of the tank, but contrary to expectation those from the high hydrogen ion experiments (pH 6.4, *C*) also choose this end. None of the other larvae reared in any of the later stocks or experiments choose this low hydrogen ion concentration when there is water of a higher acidity available.

In stock 4 the eggs were raised to hatching at Put-in-Bay and so began hatching a few hours after they were put into the experimental waters. These therefore *passed the time of heart and blood formation in the lake water*. Animals hatched on the 14th and 16th of March were kept in the experimental waters, and gradients run with them on the 17th and 22d. Nearly all of these showed the characteristic exploration of the whole tank for from three to five minutes then a preference for the acid end of the tank. The turning back occurred at a very definite pH and the avoidance was from either end, for often when an animal entered the unfavorable water it stayed there turning back from the changing point to again enter the unfavorable

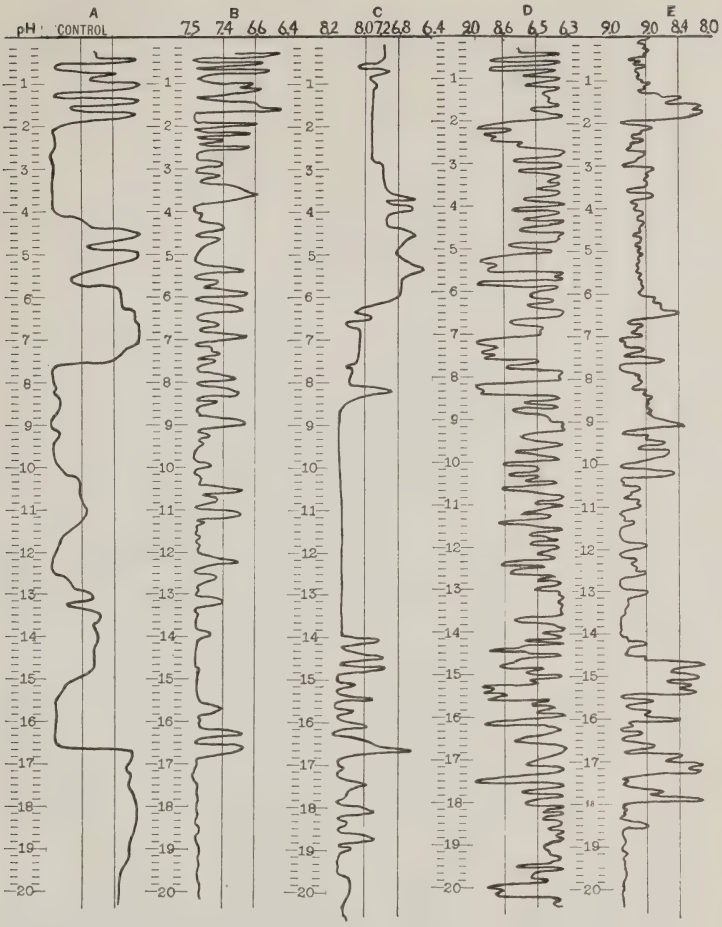

FIG. 3. Reaction of larvae of the four stocks to a permanent gradient. A long, narrow tank was used with water of the desired pH entering at each end, making a permanent gradient for the entire period of the experiment. The fish swam freely in the tank for the time recorded in the marginal figures. **A,** control larva from stock 2. **B,** larva from stock 2 raised through the period of heart and blood formation in a pH of 8.6 to 9.0. **C,** larva from stock 2 raised through the period of heart and blood formation in a pH of 6.4. **D,** larva of stock 4 raised in lake water, but hatched in pH of 6.4. **E,** larva of stock 4 hatched in a pH of 6.4 and given a choice of 8.0 to 9.0.

end several times before it escaped to the more favorable water. We therefore find the point of preference clearly marked. The fish hatched and kept in water of a pH of 6.3 show a preference for the acid end of a gradient (fig. 3 *D*). Those hatched and kept at 9.0 at first seemed to choose the 9.0 end of the gradient but they gradually worked down into the acid end and stayed there.

Wells ('15) states that if a gradient is entirely confined to pH above neutrality, 8.0 to 9.0, that fish will choose the more alkaline end even though preferring an acid pH when given a choice of 6.4 to 9.0. I found that this was true of the whitefish larvae (fig. 3 *E*). The larvae hatched in acid water (pH 6.4) chose more exclusively the part from 8.8 to 9.0 than those hatched in 9.0 water, although the latter turned at 8.4 and spent most of their time in the 9.0 end. Yet both would normally choose the acid end of a gradient from 6.4 to 9.0. Wells believes that fish are avoiding pH 8.0, and indicates that if given a choice of pH 8.0 and 9.0 they will choose the latter. This seems to be a general reaction. Plankton studies of vertical distribution show the smallest number of individuals at the thermocline (pH 8.0) with increasing numbers each side of it in either acid or alkaline waters.

The whitefish larvae reared through the period of heart and blood formation in experimental waters which were high in carbonate, and both quantitatively and qualitatively different in salt content generally from the lake waters from which the eggs were taken, behave in a gradient differently from fish reared in lake water but hatched in experimental waters. This difference lies in the direction of breaking up the preference, otherwise shown, for acid rather than alkaline waters, causing all larvae to prefer a pH of 7.2 to 8.2 (regardless of the pH of the rearing waters). Gilbert ('18) and Snyder ('23) have shown that the salmon return to the tributary in which they were *hatched*. These experiments suggest that the chemical condition of the water during heart and blood formation modifies the reactions of fishes. The salt content and the hydrogen ion concentration of streams up which salmon run differ (Van Winkle '14). These experiments suggest that the solution of these migration problems may be comparatively simple.

Summary and Conclusions

1. Hydrogen ion concentrations favorable for fertilization are too high for later development. Optimum hydrogen ion concentration is gradually lowered as the embryo becomes older.

2. The most sensitive stages in whitefish development seem to be the first cleavages and early gastrulation.

3. Fry hatching at one, two, and four months after spawning differ in size of body but not in size of yolk; those hatching at four months are 4 to 6 mm. longer than those hatching earlier. The later fry also live longer than those hatching earlier, in spite of having the same amount of yolk available.

4. Eggs raised through the period of heart and blood formation in water high in carbonates and differing from their normal environment show a different type of gradient reaction to hydrogen ion concentrations. Such larvae raised in both acid (6.3) and alkaline water (9.0) choose the alkaline end of the gradient when 6.3 to 9.0 is offered.

5. When exposed to water with a pH of 6.3 obtained by adding CO_2 directly, the larvae died earlier in the acid water than in the alkaline with a low oxygen content. A high oxygen content antagonizes the CO_2 present, prolonging the life of the larvae.

6. In a gradient of 8.0 to 9.0 larvae of both acid and alkaline hatching environment choose the alkaline end (8.8 to 9.0).

I wish to thank Dr. V. E. Shelford, at whose suggestion this work was undertaken, for his helpful advice and kindly criticism during the course of the investigation.

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VITA

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